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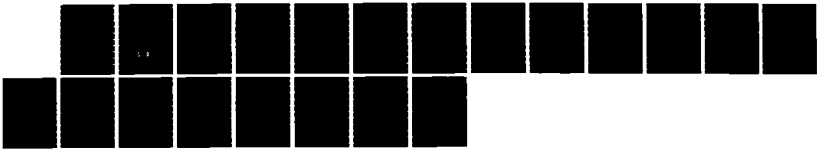

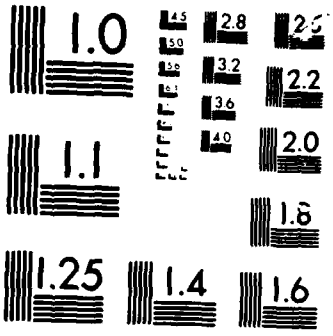
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AD-A168 937

Workshop Conference on Growth Factors in the Nervous System

Claire E. Zomzely-Neurath

June 10, 1986

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The scientific program of this workshop covered four topics: glial growth factors, neurotrophic factors, nonprotein factors, and factors affecting nerve growth in muscle. This report summarizes the presentations given under those topics.

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CONTENTS

WORKSHOP CONFERENCE ON GROWTH FACTORS IN THE NERVOUS SYSTEM

1 INTRODUCTION

This workshop conference was held at the Culpepper Conference Center, Leed's Castle, Kent, UK, from 24 to 26 March 1986. The meeting was sponsored by the Office of Naval Research, London; the Motor Neuron Disease Association; the Wellcome Trust and Muscular Dystrophy Group of the UK; and Fidia Research Laboratories, Italy. The invitation-only meeting was hosted by Dr. Frank Walsh, Department of Neurochemistry, Institute of Neurology, London, UK. The location of the meeting was excellent since the Culpepper Conference Center, Leed's Castle (a trust designated specifically for biomedical research conferences), enabled the participants to engage in informal exchanges in addition to the formal program. Furthermore, since all the participants were housed at Leed's Castle, the speakers did not have to adhere to a rigid time schedule and essentially unlimited discussion time was available.

The purpose of this small, focused workshop-conference was to bring together scientists engaged in research on growth factors in the nervous system. Mitogenic and nonmitogenic growth factors are critically important in nervous system function and maintenance. In addition to the role these factors play in neuronal development and function they appear to be crucial to nerve regeneration and wound healing, an area of interest to the Navy. The number of growth and neurotrophic factors identified in the brain has increased greatly during the past few years. As most groups use different systems for factor analysis, this conference was designed to critically assess potential similarities between growth factors and to provide a forum for discussions of the current state of this area of research as well as future prospects for research. Appendices 1 and 2 provide a list of participants and the scientific program respectively.

The Scientific program was divided into four sessions: (1) glial growth factors, (2) neurotrophic factors, (3) nonprotein factors, and (4) factors affecting nerve growth in muscle. The following sections deal with a summary of some of these topics presented at the conference.

For additional information contact Dr. Frank Walsh, Department of Neurochemistry Institute of Neurology, the National Hospital, Queen Square, London WC1 3BG, UK.

2 GLIAL GROWTH FACTORS

Astrocyte-derived Growth Factors

M. Raff (University College, London, UK) presented some of his recent studies on the role of astrocyte-derived growth factors in controlling the time of oligodendrocyte differentiation. The glial cells of the nervous system consist of two types of cells, astrocytes and oligodendrocytes. The astrocytes can be identified by the presence of the marker termed glial fibrillary acidic protein (GFAP) and oligodendrocytes by a galactocerebroside surface marker using antibodies to these compounds. Raff, using the rat optic nerve for his studies, has found that there are actually two types of astrocytes, termed simply I and II, both of which are GFAB positive but differ in developmental time. Raff found that the embryonic O-A2 progenitor cells give rise to type II astrocytes 14 days postnatal, but never to type I. Another astrocyte precursor cell develops into type I. The progenitor cells can also produce oligodendrocytes over a prolonged period. However, in tissue culture (i.e., *in vitro*) differentiation takes place in 2 to 3 days into oligodendrocytes or astrocytes. Raff posed the question as to why the *in vitro* results differed from the *in vivo*. One possibility was that the nerve axon which is absent in the tissue culture preparation was required. However, sectioning of the optic nerve did not have any different results from control. Raff found that the type I astrocyte produces a factor that prevents premature differentiation as occurs in culture. If type I astrocyte monolayer is added to the tissue

culture of dispersed embryonic cells, the oligodendrocytes do not appear for several days, just like the intact nerve *in vivo*, and continue to divide slowly. If the source of the factor is removed (i.e., type I astrocytes) the embryonic progenitor cells make oligodendrocytes right away. Thus, Raff believes that the growth factor secreted by type I astrocytes is a cell division counting clock. The concept that growth factors control differentiation is a new idea. At this time, the function of astrocytes is unknown whereas it is well established that oligodendrocytes make myelin. However, type II astrocytes are found only in myelinated fibers. Raff is presently engaged in isolating this differentiation factor from type I astrocytes and is carrying out studies to determine the function of type II astrocytes.

A. Fontana (University Hospital, Zürich, Switzerland) discussed intercellular communication between astrocytes, T cells, and macrophages within the brain. He found that Interleukin-I (IL-I), a factor that activates T-cells of the immune system, is produced by activated astrocytes but does not know if type I or II astrocytes are involved. These astrocytes also produce a factor that appears to be a growth factor for macrophages which Fontana thinks is either Interleukin 2 or 3. Fontana presented preliminary data; he is actively pursuing studies of those factors produced by astrocytes which appear to modulate cells of the immune system.

Control of Growth and Differentiation in Normal and Tumor Cells

M. Noble (Department of Clinical Neurology, University of London, UK) dealt with division and differentiation in normal and neoplastic central nervous system (CNS). Noble has found that platelet-derived growth factor (PDGF) is the only known growth factor that can replace astrocyte-conditioned medium for glial cell growth in culture. Noble does not believe, however, that astrocytes make PDGF as he found no detectable PDGF using a complementary DNA (cDNA) probe to PDGF. It is known that PDGF is made by endothelial cells. Antibodies to PDGF do not block the effect of an astrocyte-conditioned medium on cell division. Thus it appears that the growth factor present in astrocytes is not PDGF. Noble is presently trying to isolate this astrocyte-derived growth factor.

Noble and his group are also carrying out studies of human brain tumors, of which 50 percent are gliomas; i.e., glial cell tumors. About 10 percent of malignant astrocytomas are poorly differentiated. There is no detectable glial fibrillary acidic protein (GFAP) in tumors producing antibodies to GFAP as well as no expression of glial specific markers in 90 percent of these tumors. About 10 percent of the astrocytomas are positive for fibronection and also positive to GFAP and A_{285} (a glial antigen antibody). Most of these tumors grow very slowly, but if grown in tissue culture containing astrocyte-conditioned medium, cell division and differentiation takes place.

During the past four months, Noble and his group have been making designer cell lines using cells from the corpus collosum with the addition of murine leukemia virus (large antigen of SV_{40} virus). Foci are obtained and cloned to characterize the system. It was found that a second oncogene could be added to the system to study control of differentiation, and questions could be asked about control of growth. Noble was able to obtain differentiation with maturation-arrested cells using defined conditions. Depending on the different media used, he could obtain GFAP positive cells as well as type I astrocytes that were GFAP negative. Noble is also trying the Ras oncogene in his system in order to confer independence of growth factor control. He found that if he used Mic oncogenes he was able to obtain colonies in culture that looked like well-differentiated oligodendrocytes.

These are all preliminary studies; Noble and his group are actively investigating the factors that control growth and differentiation in tumor cell lines as well as in normal cells. He is also trying to obtain neuronal cell lines as

well as mixtures of glial and neuronal cultures. Information about factors that can affect differentiation in tumor cell lines will be applicable to basic knowledge about normal cells because tumor cells are essentially undifferentiated and control of growth is absent. Although the neuron is the cell concerned with the most important functions of the brain, i.e., neural transmission, there is some evidence that factors produced by glial cells may also affect the activity of neurons.

3 NEUROTROPHIC FACTORS

Brain-Derived Growth Factors

Y. Barde (Max Planck Institute for Psychiatry, Martinsried, Munich, West Germany) has focused his research on obtaining neurotrophic factors in the CNS. Nerve growth factor (NGF), the well-characterized neurotrophic factor, acts primarily in the peripheral nervous system with little or no effect on CNS neurons. Therefore, Barde and his group had to look for unknown factors in the CNS. Barde developed a bioassay using dissociated sensory ganglia from the chick (the sensory ganglia are connected to the CNS). He was able to culture pure neurons without requiring nonneuronal cells. The neurons die very quickly unless some factor is added, and Barde found that the addition of a brain extract kept the neurons alive. Using pig brain, Barde obtained a factor in fairly large amounts which he termed brain-derived neurotrophic factor (BDNF). Using acid precipitation, carboxymethyl cellulose (CM) hydroxyl apatite, and hydrophobic (acetylphenyl sepharose) and reverse phase chromatography, Barde obtained 10 µg of BDNF from 20 kg of pig brain. This was a good yield since growth factors are present in only minute amounts in nervous tissue. Although the specific activity was fairly low, only a very small amount was required to produce an effect in his bioassay procedure. The BDNF is a highly basic protein (isoelectric point [IP] greater than 10) as is NGF. The molecular weight of BDNF is 14,000, about the same as cytochrome C. Amino acid analysis of BDNF showed the absence of proline, tryptophan, and cysteine but BDNF contains two methionines which aided in sequencing this factor.

Barde is now sequencing BDNF in the picomole range and will use synthetic oligonucleotides based on the amino acid sequence to prepare cDNA and to clone it. In this way, it is possible to obtain larger amounts of BDNF using an expression vector in recombinant DNA procedures, thus eliminating the tedious and time-consuming isolation of BDNF from the brain. In studies of the specificity of BDNF, Barde found that sympathetic ganglia react to NGF but not to BDNF, while neither were effective with ciliary ganglia. Sensory ganglia from the neural crest epidermal plaque of different origins from sympathetic and ciliary ganglia responded to BDNF but at different levels of reaction. Mesencephalic neurons (from trigeminal nerve innervating muscle) are unique, homogeneous and responded very well to BDNF with 50 percent survival of neurons as well as being negative to NGF. Barde also carried out some studies using retina to see if it would be a target for BDNF action. Using phase contrast microscopy between control and BDNF-treated retina at embryonic day 17, no effect was observed, but he did see a difference from control with Thy 1-antigen. In an embryonic tissue culture system, however, a large response was obtained with BDNF after 2 days in cultures with no effect with NGF. Addition of BDNF can maintain neurons in culture for at least 2 weeks but does not cause mitosis (i.e., cell division) as does the well-known fibroblast growth factor (FGF). In the developing rat retina, Barde found that BDNF is required from the start of embryogenesis in order for the retinal axons to project towards the target cells by embryonic day 17. However, there is little effect of BDNF on neuronal survival postnatally. At this time, Barde does not know if BDNF acts on neurons other than retina and also has not studied the effect of BDNF, if any, on astrocytes, as suggested by Raff.

A. Leon (Fidia Research Laboratories, Abano Terme, Italy) presented her

recent unpublished work on the isolation of a neurotrophic factor from bovine striatium. Her factor appears to be similar to the BDNF factor isolated by Barde. During the course of the meeting, Barde and Leon arranged to exchange detailed information about their respective factors, and Barde will check Leon's antibody to her factor against his BDNF. It appears that although the two factors are very similar in physical-chemical characteristics they are probably not identical and the check with Leon's antibody will give a definitive answer, especially since she has three monoclonal antibodies raised to different fragments of her factor.

Leon used dissociated mouse mesencephalic neurons from embryonic day 13 for the bioassay used in following the purification of her neurotrophic factor. Since the factor was isolated from striatum which consists primarily of glial cells, it is a glial-derived factor. However, it is known that glia secrete neurotrophic factors that sustain neuronal survival, repair, and growth. In the bioassay, neuronal growth was checked with antibody to neuronal filament protein which is present in axons of the neuron. Electrical activity of neurons was also checked; survival of dopaminergic neurons was tested by measuring dopamine uptake and general neuronal survival by DNA content.

The Leon neurotrophic factor was isolated by acid precipitation at pH 4.5 of a homogenate of bovine caudate nucleus, and dialysis with 8000 molecular cutoff membrane; this was followed by Sephadex G-150 and CM-cellulose chromotography and fractionation by high pressure liquid chromatography (HPLC). The active fraction was highly basic (I.P. of >10) and molecular weight of 14,400. Addition of factor to the tissue culture system gave a dramatic effect on neuronal growth in the bioassay system. The final purification was 85,000 fold. The chemical characteristics of the factor are: (1) basic and (2) trypsin sensitive and heat labile. Its biological parameters are: (1) increases neuronal cell survival and (2) increases neurite outgrowth. Also, this new factor was definitely determined not to be NGF. Leon collaborated with F. Walsh, Institute of Neurology, London, who was able to make three monoclonal antibodies (Mabs) to Leon's brain factor. These Mabs were active in pulling out the brain factor from the supernatant fraction used for isolation of the factor. At this time, Leon does not know if her factor is present in brain areas other than mesencephalic neurons. These were used for the bioassay since they project into the striatum. Barde stated that he was unable to obtain Mabs or even polyclonals to his BDNF even though he and his group have considerable expertise in this area. Leon's original aim was to isolate a neurotrophic factor specific to dopaminergic neurons but her brain factor is not specific to any single type of neuron. Leon is now in the process of sequencing the factor and preparing cDNA for *in situ* hybridization studies. She still wants to try to get a factor specific to dopaminergic neurons but these neurons are so few that she must try to enrich the mesencephalic neuron assay for doperminergic neurons in order to have an assay sensitive enough to pick up a specific factor.

Neurite Extension Factor

The isolation of another new neurotrophic factor was reported by D. Kligman (National Institute of Health). His goal, like that of Barde and Leon, was to obtain a neurite extension factor from CNS neurons. The heterogeneity of the CNS compared to the peripheral neurons system (PNS) makes it especially difficult to isolate factors from CNS material. Kligman used 7-day chick embryo cerebral cortex, dissociated, trypsinized, and plated in defined medium for his bioassay. Tetanus toxin was used to identify neurons in the tissue culture. Even with his partially purified neurite extension factor (NEF) he was able to find neurite extension at 60 hours following addition of NEF to his bioassay system. Kligman used bovine brain for purification of NEF. He found the activity of the factor

was stable to heating at 90°C for 5 minutes and since most proteins are denatured at high temperature was then able to use heat treatment to get rid of large amounts of other proteins during the isolation procedure. Kligman's factor in contrast to BDNF and Leon's factor is acidic, binding to diethylaminoethylcelulose (DEAE) at neutral pH. Thus, the crude supernatant fraction after heat treatment was eluted using a salt gradient of 0.15 to 0.35 M in tris-HCl buffer using DEAE chromotography. Reverse phase HPLC on propanylsilica was used for purification. NEF activity is eluted as a sharp symmetrical peak long after all other proteins are eluted. Bovine serum albumin or gelatin (nonspecific protein) was used to prevent binding of NEF to solvents. These proteins were negative in the bioassay. The molecular weight on electrophoresing gels was 6000 in the presence of ß-mercaptoethanol (i.e., subunit) and 26,000 in the absence of the reducing agent. Analysis of the amino acid composition showed no proline, a high content of the acidic amino acids glutamic and aspartic, and was very similar to the amino acid composition of S100 protein, which is a calcium modulator in the nervous system. NEF also has a blocked amino terminal as does S100B. Kligman has raised polyclonal antibodies to NEF in rabbits and, using this antibody, found that it labels astrocytes and appears at postnatal day 10 during development. NEF antiserum also recognizes S100 protein with equal affinity to NEF. Kligman found that NEF was active on rat hippocampal neurons, with sprouting occurring 4 to 6 hours after addition of NEF. The neurite-promoting activity was found to be twice as high as that of NGF, the first neurotrophic factor identified several years ago by Levi-Montalcini. When Kligman tested S100B in his bioassay system, it was inactive. Furthermore, although NEF and S100B are identical in primary structures, the tertiary structure is different. Thus, NEF and S100B are similar but not identical proteins. Kligman is now cloning the cDNA for NEF for further characterization and localization of this new CNS neurotrophic factor.

Sensory Neuron Growth Factor

R. Lindsey (Sandoz Institute for Medical Research, London) discussed developmental and regional differences in the growth factor requirement of sensory neurons. He emphasized that specificity has not been obtained as yet in CNS factors partly due to the difficulty in obtaining even nonspecific factors from the markedly heterogeneous CNS tissue as compared to the more simple peripheral nervous system (PNS) tissue. He compared the effect of NGF on neurite outgrowth of dorsal root ganglia (PNS) and nodose ganglia of brain. The latter was unresponsive to NGF whereas a marked effect of NGF was seen on dorsal root ganglia, as expected. He found that there were no receptors for NGF on nodose ganglia cells (cranial ganglia), which accounted for the lack of any effect of NGF. Surprisingly, Lindsey obtained growth of sensory neurons in culture by addition of an extract of chick liver with the neurons surviving for 10 to 14 days in culture. NGF, on the other hand, had no effect on the growth of sensory neurons. The growth factor from liver comes from the nerve afferents in liver and not from liver tissue per se. Thus the factor is derived from nerve tissue. Lindsey has been able to separate out neurons from dissociated adult cells and, with replating, obtains survival for 27 days. NGF and BDGF both show an effect in 1-day adult neuronal cultures but after 7 days and up to 21 days are no longer required for survival. The growth is the same as that of controls without addition of NGF or BDGF. Lindsey is investigating when the switch from dependence to independence occurs. With the addition of astrocyte supernatant, there is 50 percent survival of nodose neurons but he does not know if astrocytes contain BDGF or some other factor. Also, at this time, Lindsey does not know if his factor from liver (nerves) is the same as BDGF or other brain factors; he is presently purifying his factor from liver to find

out whether or not he has another new neurotrophic factor.

Nerve Growth Factor

G. Dickson (Institute of Neurology, London, UK) has been studying active nerve growth factor (NGF) expression in PC12 cells (pheochromocytomas from chromaffin granules of adrenal medulla). The neural crest can become either adrenergic or noradrenergic neurons and NGF appears to be involved in determining which pathway is followed. Addition of NGF to PC12 cells in culture leads to neurite outgrowth and cessation of mitosis. The acute response to NGF in PC12 cells leads to increased transcription for β-actin, ornithine decarboxylase, and neurofilament protein as well as activation of tyrosine hydroxylase. Dickson also found increased Thy-1 expression. He then looked at messenger RNA (mRNA) levels of several neural components after addition of NGF to PC12 cells, using cDNA probes. He found increased mRNA levels for N-68 (neurofilament protein), Thy-1, γ enolase (neuron-specific γ-enolase) N-CAM, β-actin, and glycerol 3-phosphate-dehydrogenase. The highest levels of mRNA (8-fold increase) were found at 3 days after addition of NGF, with levels remaining constant thereafter following a transient slight decrease. Induction by NGF is thus a transcriptional event. When NGF was withdrawn from the tissue culture there was no change in mRNA levels up to 7 days after removal of NGF, demonstrating that NGF is not required for maintenance of mRNA levels and that the phenotype is stable. The increase in mRNA was concurrent with neurite outgrowth. However, if neurite outgrowth is prevented, i.e., by suspension of cells, no change in mRNA levels is seen. Dickson concluded that NGF responses are independent of neurite outgrowth and that the activated phenotype does not require continued NGF stimulation. The primary inductive events mediated by NGF involve activation of Thy-1 and NF-68 genes. NGF may influence maintenance of the overt differentiated state via membrane potential, nutrient uptake, and growth cone activity according to Dickson. He is presently using PC12 cells to study the control of gene expression; for example, DNA methylation.

L. Reichart (University of California, San Francisco) and his group are studying factors affecting nerve cell survival and neurite outgrowth. The presentation was given by D. Shelton from Reichart's group as he was unable to attend the conference. Reichart et al. are using NGF, which is required for the maintenance of sympathetic and sensory neurons *in vivo*, to find out where NGF is synthesized. They are using a recently cloned NGF provided by W.J. Rutter, University of California, San Francisco. The clone contains 900 base pairs and all the mRNA code. The detection system, using Northern blot and agar gel electrophoresis with hybridization of the RNA using their labeled clone, was optimized, so the level of detection was sensitive at 20 centograms instead of the usual picogram level. Using Southern genomic blot, and genomic DNA from rat, cow, mouse, and dog, these researchers found the same amount of hybridization in each species studied, showing that the assay could be used for species other than mouse, for which the assay was initially developed. Tissues with sympathetic innervation were studied and no tissue was found that did not contain NGF mRNA. Tissues with a high levels of sympathetic innervation such as iris and spleen contained high levels of mRNA whereas a tissue with low innervation such as liver had low NGF mRNA. Reichart et al. also measured norepinephrine levels, and when plotted against levels of NGF mRNA a strong positive correlation was found between the amount of norepinephrine in a tissue and the amount of NGF mRNA. However, this is not a direct correlation because the same curve is obtained if dopamine β-hydroxylase levels are plotted against norepinephrine levels. Thus, the increased NGF mRNA is more a measure of sympathetic innervation. When the PNS is assayed for NGF mRNA, as in the sciatic nerve and dorsal root ganglia, high levels of mRNA are found. The levels of mRNA are actually minute--in fentograms per microgram of total RNA. Thus, it was

possible to carry out all these studies with the use of cloned NGF cDNA.

Neurons in the CNS (cholinergic neurons of basal forebrain nucleus) responded to NGF by inducing choline acetyltransferase activity. NGF mRNA was also measured in various areas of brain of rabbits. The hippocampus and pyriform entorinal neocortex were found to contain the highest levels of NGF mRNA. The septal area and striatum contained low levels of NGF mRNA, with the lowest levels in the cerebellum. Reichart et al. found that NGF has more subtle effects in the CNS as compared to the PNS and think that there may be as yet undescribed NGF-responsive neurons in brain.

Reichart et al. carried out studies using the rat iris to find out what controlled NGF. Denervated iris in explant culture exhibits increased NGF but no increase in NGF mRNA. On the other hand, in an explant of iris which is not denervated a large and rapid increase of NGF mRNA is found during a few days in culture. An 8- to 22-fold increase in NGF mRNA is found with a time course similar to an increase in NGF level and a peak at 6 to 24 hours. Since serum was used in the culture and *in vivo* the iris is not exposed to serum, it was necessary to rule out the serum factor. Reichart et al. found that culture of the iris explant in serum-free medium had the same effect of increased NGF mRNA as in a culture containing serum. When the iris explant was cultured in its own aqueous humor, however, there was no induction of NGF mRNA, indicating that some factor is present in aqueous humor which maintains the NGF mRNA at a constant level. Reichart et al. are presently trying to obtain this factor.

Trophic Factors in Ciliary Ganglia

M. Manthorpe (University of California, San Diego) presented his work on protein influence on survival and growth of ciliary ganglia neurons. There are two populations of neurons in ciliary ganglia, both of which are cholinergic neurons and are subject to developmental neuronal death. In other words, more neurons are made than are required for innervation so that by 8 to 14 days of development there is a 50 percent loss of neurons. Manthorpe used 15-day chick embryo extract as a bioassay. As the extract is diluted, the number of neurons with neurite outgrowth decreases markedly. To assay cell death, a tetrazolium dye was used. The dye is taken up by the mitochondria of the cells, and mitochondria stain blue. Prior to cell death, the cell ruptures and the blue mitochondria are released into tissue culture. In the absence of a trophic factor there is little staining, but in the presence of factor, intense staining is seen. By eluting the blue stain, the intensity (optical density) of the dye was measured and was found to be proportional to the number of surviving neurons. This is a simple procedure developed by Manthorpe which eliminates the necessity of counting neurons, a very tedious procedure. The method is fast, accurate, and a low number of cells is required. The limitations are: (1) possible presence of compounds which reduce tetrazolium, i.e., loses blue color; (2) it measures all living cells so pure neurons only are required; (3) presence of dehydrogenase activity renders the intensity of dye disproportional to cell number.

Manthorpe has found two trophic factors in ciliary ganglia. Sources for the factors are cocultured cells, cell-conditioned media, and tissue extracts. The trophic factors are also present in rat sciatic nerve extract. He used DE-52 ion exchange chromatography, sucrose gradient fractionation, and preparative SDS-PAGE electrophoresis followed by reverse phase HPLC, to purify the factor. The factor from chick eye extract has an IP of 5.0 and molecular weight of 20,000 to 25,000. From rat sciatic nerve, the PI is 5 to 8 and molecular weight is 28,000. Manthorpe is presently sequencing the factor and plans to prepare antibodies to the factor as well as to make cDNA, using synthetic oligonucleotides based on the amino acid sequence, and to clone cDNA for further studies.

Manthorpe has isolated a neurite-promoting factor (NPF) from rat Schwannomas (a Schwann cell tumor) using DE-52

chromatography, cesium chloride equilibrium gradient centrifugation, and sucrose density gradient centrifugation. He obtains 50 to 100 µg of purified material which is very potent at 10^{-11} to 10^{-13} molar range for half maximal activity. The NPF is large, about 10^6 molecular weight. He has found that it is a very active glycoprotein which binds to ConA columns. NPF appears to be similar to extracellular matrix protein although components of this protein such as heparin and hyaluronic acid had no activity in the bioassay. On the other hand, laminins from human, mouse, and rat showed high activity in the bioassay, indicating that perhaps laminin is a component of NPF. Manthorpe is presently investigating this possibility and is also sequencing NPF and plans to clone NPF.

Neurite-Promoting Factors for Spinal Neurons

C. Henderson (Pasteur Institute, Paris, France) is investigating neurite-promoting factors for spinal neurons (motor neurons). He is trying to find out if there are factors in muscle that would promote survival of motor neurons, i.e., a muscle neurite-promoting activity. Using conditioned medium from culture of chick 11-day leg muscle (skeletal muscle) Henderson obtained neurite outgrowth of chick neural tube cultures. The percent of cells with neurite outgrowth was proportional to the amount of extract added to the culture. Henderson found that the muscle factor was trypsin-sensitive with a molecular weight of 20 to 50,000; 11-fold enrichment with high specific activity was obtained. He used a high-speed supernatant fraction of chick leg muscle from 3-day post-hatch chicks rather than conditioned medium in order to obtain enough material for isolation of the factor. Henderson found a developmental regulation of the trophic activity with a 10-fold increase after 3 to 4 days with activity almost back to normal levels by 10 days. An extract of denervated muscle yielded a 15-fold increase in neurite-promoting activity in 3 days as measured by bioassay. However, Henderson said that he could not be certain that his factor was derived solely from muscles because denervation leads to several effects in muscle and surrounding tissue. Henderson stated that he only could be certain that it is a muscle factor by preparing a cDNA probe to the factor and testing this probe. Gel analysis of Henderson's factor yields three bands on electrophoresis. However, he found that the neurite-promoting activity was associated with one narrow band at 25,000 molecular weight. He is now attempting to prepare Mabs to this factor.

Henderson has also carried out some preliminary studies of human diseases that result in a selective loss of motor neurons in the spinal cord. Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is one example; another, that occurs in children, is Werdning-Hoffmann (WH) disease, a genetic disease that leads to a selective loss of motor neurons during the first few years of life. Using extracts of biopsy tissue of ALS and WH patients Henderson found that neurite outgrowth of chick extract-treated neurons was inhibited by extracts of diseased human muscle. The inhibition occurred at very low protein concentrations of the human muscle extract (about 1 µg). Henderson is trying to characterize this inhibitory substance.

4 NONPROTEIN FACTORS

Cell Surface Carbohydrates

R. Schnaar (Johns Hopkins University, Baltimore) discussed a possible role for cell-surface carbohydrates in neuronal recognition. Cell-surface carbohydrates appear to be required for cell-cell interaction and can be potential ligands for cell-cell recognition. There is fairly good evidence that the directive influences in neuronal connectivity include: (1) impulse specificity, (2) mechanical contact guidance, and (3) chemoaffinity (soluble factors, cell-cell touching recognition). Axons can find their targets; extension of axons proceeds in a geometric way. When Schnaar used antibody to neural adhesion molecule

(N-CAM) in retina, he found that some axons go to their proper orientation on tectum while some go in other directions but then turn 90° and go on to tectum (target cell). If hepatocytes and heart myocytes are dissociated and mixed, the hepatocytes go to hepatic cells and the heart myocytes to heart within a few minutes.

Schnaar is trying to identify a class of molecules involved in cell-cell adhesion such as N-CAM. Every cell in vertebrates is coated with glycoprotein, proteoglycans, and lipids with complex carbohydrate structures. There is also structural diversity as there are a large number of primary structures made from relatively few building blocks. Apparently there are receptors on the cell surface to recognize and bind carbohydrates. For example, mammalian hepatocytes recognize galastose, N-acetyl galactose, and fucose. If the inappropriate sugar is added, there is no effect on cell adhesion. Schnaar developed an assay to measure the strength of cell adhesion. The saccharide to be tested is added to cells in gel. After adhesion occurs the plate is immersed in solution and a centrifugal force is applied. Weak adhesive compounds are dissociated from binding to the cell surface; i.e. sugars that are not recognized by cell surface receptors. The adhesion is temperature-dependent. At 4°C and 37°C, adhesion is the same for all sugars but when the centrifugal force is increased sugars are dissociated at 4°C but adhere strongly at 37°C. This adhesion can be blocked by metabolic inhibitors, so adhesion appears to require metabolism. However, if soluble sugar is added, then adhesion can be reversed and reversible adhesion is followed by metabolic change.

Schnaar used retinal cells dissociated with trypsin to obtain single cells and tested their ability to recognize particular carbohydrates. It was found that simple sugars do not support adhesion of retinal cells but neural retinal gangliosides did support adhesion and to a much greater extent than other lipids. Gangliosides are present in high concentrations in the nervous system and face the outside of the cell with a hydrophilic carbohydrate portion. Schnaar finds some specificity of gangliosides when he measures strength of adhesion, so it is not just a charge binding. He thinks there are ganglioside receptors on the cell surface. The ganglioside adhesion was also found to be temperature-dependent. Schnaar has preliminary evidence that phosphoproteins may also be involved in neural cell adhesion as, temporally, phosphorylation follows strengthening of cell adhesion.

Gangliosides and Neuritogenesis

P. Doherty (Institute of Neurology, London, UK) is studying the specificity of ganglioside effects on neuritogenesis. He finds that gangliosides added to cultures of chick dorsal root ganglia lead to neurite outgrowth. Doherty used Mabs to neurofilament protein to quantitate neuritogenesis (neurite outgrowth) in the cultures. The questions he posed were: (1) are gangliosides themselves trophic for neurons, (2) at what levels do gangliosides and NGF interact to promote neural recognition, (3) do individual gangliosides differ in specificity, and (4) does the effect of exogenous gangliosides reflect the function of their endogenous counterparts. Doherty found that dorsal root ganglia cultured for 5 days resulted in an increase of NF (neurofilament protein) as the NGF concentration increased. If anti-NGF serum was added to the culture there was no survival of neurons by day 5, but if NGF plus GM were added the effect was greater than NGF alone. Thus, there appeared to be a direct interaction between the two agents. If cultures are grown with NGF and at day 2, NGF is removed, there is no effect on growth from days 2 to 5 as measured by the NF assay. Also, antiserum to NGF had no effect on abolishing NGF response if added at day 2. Doherty thinks that maintenance of the NGF effect may be due to factors in nonneuronal cells in the culture. Testing of GM, as well as other gangliosides (GT1b, GQ1b, etc.) showed that they will affect neuritogenesis independently of NGF and that the effect was different for each of the

gangliosides. Also, antibodies to NGP do not inhibit the ganglioside response.

To investigate the role of the various gangliosides, Doherty has prepared Mabs to a series of gangliosides. Doherty has found that Mabs prepared to GMI can modulate neuroblastoma differentiation. However, he found no effect of Mabs to GMI on NF expression in primary cultures of embryonic day-7 chick dorsal root ganglia. The reasons for this result could be that (1) no GMI is in the membrane, (2) GMI is present in membrane but unavailable to Mabs, or (3) the Mabs are no good. To investigate these possibilites, Doherty treated the cells with neuraminidase and found synthesis of GMI as well as a good antibody-binding signal. Also, choleratoxin which binds to the membrane also binds GMI very well. So the Mabs do recognize GMI if present in the cell membrane, and Doherty thinks that there may be different compartments for GMI or that endogenous GMI differs from exogenous GMI. Doherty is presently studying whether GMI stimulation of neuritogenesis can occur in the absence of nonneuronal cells.

5 FACTORS AFFECTING NERVE GROWTH IN MUSCLE

Regeneration of Peripheral Motor Neurons

D. Kuffler (Biocenter, Basel, Switzerland) presented a model system using the frog to study regeneration of peripheral motor axons. He is looking for cues from the motor neurons to aid them in finding their way to the target muscle. To readily see the organization of nerves, Kuffler uses cutaneous pectoral muscle of frog. If the nerves are crushed, one can follow regeneration of the motor nerves. Kuffler finds evidence of mechanical guidance by Schwann cells of the basal lamina. He posed the question of whether the cues for directing regeneration of the motor axons came from the Schwann cells or from the degenerated neurons after nerve crush.

Kuffler investigated the effect of removal of each of the cell types in his system. If the muscle fiber was damaged but the nerve left intact, 40 percent of old synaptic sites were reinervated so the target (i.e., muscle) was not essential. If the entire surface of the muscle was frozen and then the muscle allowed to regenerate, fibers grew down the naked basal lamina and reinervation resulted. If the entire area is frozen to prevent muscle regeneration only 5 to 10 percent of the original sites become inervated, indicating a role played by the nerve tube as well as by muscle. Kuffler then cut out the cutaneous pectoral muscle but left the nerve fibers intact. A connective tissue sheet was then laid down by the frog to take the place of the pectoral muscle. Thus, Kuffler had an *in vivo* system mimicking an *in vitro* one and could add a variety of compounds to the connective tissue sheet to study the effects of the additions. If he uses horseradish peroxidase or silver strain, he can see the axons growing over the connective tissue sheet. There is no peripheral target, so the axons grow out in many directions.

Kuffler then measured the targeting of each axon. He found that some axons showed a highly directed pattern of outgrowth. The axons go to the satellite target through the original basal lamina using old Schwann cells and old basal laminae whereas other axons had not reached the target during the same time period. If denervated nerve is implanted as target there is only a slight reduction of the pattern of outgrowth so it is still highly oriented. If Kuffler inserts a millipore filter which traps cells but permits factors to pass through, highly directed patterns of axonal outgrowth are found. Thus the axons are recognizing a signal released by cells on the filters. Kuffler thinks that cells both of the nerve tube (Schwann cells) and muscle target contribute to directing axonal regeneration. In this respect Kuffler found that tendons have no effect, unlike muscle, on regeneration. Barde mentioned that he thinks the trophic factor is NGF. This possibility is being studied by Kuffler but he may also have a new trophic factor.

Nerve Regeneration

T. Sears (Department of Neurophysiology, University of London, UK) studied protein synthesis in cat intercostal motor neurons by electron miscroscopy (EM). He found that after chronic axotomy (no regeneration of nerves) the EM profile of the polysomes became clumped and disorganized and no longer exhibited the ordered polysome structure with the polysomes arranged on the endoplasmic reticulum. With acute axotomy (crushed nerve) the polysomes reform into their normal ordered structure as the nerve regenerates. Sears thinks that this requires a signal from the target cell (muscle). This is a new finding and Sears wants to investigate this phenomenon to find out what the hypothetical facts might be whose absence apparently prevents regeneration of nerves by inhibiting the formation of the normal protein-synthesizing system necessary to make the proteins needed for nerve regeneration.

M. Brown (University of Oxford, UK) presented some of his studies on factors controlling nerve growth in muscle. He uses the gluteus muscle from mouse for his studies as it is easy to get partial denervation and also the muscle is thin enough to get good staining. Brown finds that 6 days after denervation, sprouts of two types are seen: (1) nodal sprouts which project to denervated endplates and (2) terminal sprouts which grow out of terminals and extend towards denervated endplates. With partial denervation of muscle, there is a very vigorous response, indicating the presence of potent growth factors. If half the motor axon is removed, full recovery occurs within 10 days in the mouse. Brown listed several possibilities that could account for this effect. These are: (1) growth factors from muscle; (2) substrates (environment) that cause changes in surfaces so that nerves can grow; (3) degeneration products; and (4) transneuronal effects, i.e., some factor goes from axotomized neurons to intact nerves, resulting in changes. Brown found that in motor neurons, nodal sprouts are found only within a muscle. Muscle paralysis alone can induce both terminal and nodal sprouts, indicating that the degenerated nerve alone does not provide the factors for sprouting, i.e., that a growth factor from muscle is also involved. Also, empty sprouts need something on which to grow; e.g., endothelium, a substrate from degenerating nerve. Brown made antibodies to an extract of denervated muscle and found that he could block regeneration by injection of the antibody into the mouse. He also found that N-CAM, which is normally found only at the end plate, is synthesized in high amounts if muscle is degenerated. In a preliminary study, Brown was able to block terminal sprouting with administration of antibody to N-CAM. This indicates that N-CAM is involved in the sprouting process but is probably only one of several factors that play a role, and Brown is trying to find what these other factors are.

Recombinant DNA Techniques to Study Nerve Growth and Regeneration

F. Walsh (Institute of Neurology, London, UK) discussed his studies on the regulation of N-CAM expression in skeletal muscle. He is using an *in vitro* tissue culture system of a transformed mouse muscle cell line (G8-NIH) for his work. After 3 days in culture, muscle contractile proteins (myosin and actin) appear as well as creatine kinase (ATP biosynthetic enzyme). In this system Walsh can investigate growth, confluence, fusion, and degeneration. He is using techniques of molecular biology (recombinant DNA) and immunology to look at events at the molecular level. Walsh uses antibodies to specific markers (antigenic markers of skeletal muscle cells) for some of his studies. These antibodies are: N-CAM; 5 1H11, U13H, Thy 1, 163AS, etc. He has found that class I antigens (N-CAM, 5 1H11, U13H) are specific to cells of muscle lineage and are not expressed as, for example, in fibroblasts. The antigenic marker (24 ID5) was found to be present only in myoblasts and is the most specific marker (using Mabs) that Walsh has found so far. Most of the Mabs that Walsh has prepared appear to be human-specific, unlike

polyclonal antibodies which react with several species. In his *in vivo* studies of N-CAM in skeletal muscle, Walsh has found that: (1) developing muscle is N-CAM positive, adult is negative, and the down regulation coincides with muscle fiber development; (2) denervation reactivates N-CAM expression, renervation causes a down regulation of N-CAM, and paralysis by tetrodotoxin or botulism toxin reactivates N-CAM expression; (3) N-CAM is present at the neuromuscular junction and does not coincide with the presence of acetylcholine receptor (AChR). N-CAM is absent from fibroblasts but is present on myoblasts and myotubes. Using a complementary DNA (cDNA) probe to mouse N-CAM, Walsh looked at mRNA levels in the G8-NIH culture during development. He found that at the myotube stage, the predominant messenger was a 2.8 kilobase (kb) message plus a larger message of 5.4 kb. At the myoblast stage, the higher molecular weight mRNA was predominant with only a small amount of the lower molecular weight. At 2 days in culture a 6.7 kb message appears and by 6 to 8 days in culture, when fusion starts, the large messenger has disappeared; Walsh thinks, therefore, that this message is specific for myoblasts. At present Walsh is trying to find the proteins expressed by these developmental messages.

Walsh has been attempting to isolate the mRNA species encoding N-CAM. He cloned cDNA from human skeletal muscle into the expression vector λgt-11. He screened 5×10^5 events and was able to isolate 50 recombinants corresponding to 14 sets of individual events which have been subcloned into Puc9 and M13 for futher analysis to obtain the N-CAM gene from human skeletal muscle. Preliminary data indicates that the N-CAM gene is located on chromosome 11, which is also the location of the human Thy 1-gene. Walsh has also been able to isolate the antigen for the myoblast specific message and is trying to find the antigens encoded by the other messages during development.

Fibroblast Growth Factor Effects

S. Hauschka (University of Washington, Seattle) is studying the regulation of skeletal muscle growth and differentiation by fibroblast growth factor (FGF). Mesodermal cells committed to myogenesis go through the myoblast (replicating, undifferentiated stage) to mycotes. Postmitotic cells are differentiated and after fusion become myotubes. He is interested in finding out what causes the observed increase in the number of myoblasts during development and what turns on muscle-specific genes. Muscle cells, like neurons at differentiation, become postmitotic. Hauschka wants to find out what is involved in the postmitotic state. He is using a permanent line of mouse myoblasts which he has derived. He uses a number of different mouse strains that are spontaneously transformed. These strains can grow forever before transformation. If purified FGF is added to the culture, replication continues as long as FGF is present. Removal of FGF leads to extensive fusion within 14 hours. There are two forms of FGF, acidic (α-FGF) and basic (β-FGF) (i.e., two different genes). There is 55 percent homology between the two forms and 25 percent homology with interleukin-1 (IL-1). α-FGF has a neuropeptide-like amino acid sequence when checked by computer with all known nueropeptides. Chick 12-day embryo culture is not dependent on FGF but FGF does delay onset of differentiation. Mouse (18-day culture) is the most dependent on FGF, and in human cells there is some dependence on FGF for development. Hauschka purified α and β FGF from bovine brain to study how FGF regulates terminal differentiation of muscle cells. The mouse myoblast cell cycle is very sensitive to FGF. If insufficient amounts of FGF are added to the medium, the cells undergo irreversible decision to terminate. Addition of FGF at this stage will not start differentiation. The total cycle takes 12.5 hours. If FGF is removed from the medium, proliferation occurs with cell doubling, then plateaus. If FGF is added again, cells will continue proliferation. FGF represses the commitment to terminal differentiation via a mechanixm which is independent of cell replication. Hauschka thinks that mouse myoblasts require multiple macromolecular factors

for replication; i.e., FGF plus some unidentified components. Replication can thus be prevented independently of FGF. FGF stimulates proliferation and represses commitment; i.e., it has two effects.

Hauschka thinks that if one can understand how muscle-specific genes are activated this might give information about where the trophic factor acts and, working backwards, one can find the factor to identify a commitment gene; i.e., what makes one mesodermal cell a myoblast (muscle) or another chondroblast (bone). When fusion starts in 12 hours, a marked rise in total creatine kinase activity is seen; AchR-positive cells can be detected at 6 to 8 hours, well before fusion, but commitment occurs as early as 3 to 4 hours. Thus, Haushka has cloned the creatine kinase gene from mouse and is studying the regulation of this gene. He puts the cloned gene back into mouse myoblasts (tranfected gene). It is necessary to distinguish the mRNA-injected gene (exogenous) from endogenous on mRNA. To do this, Hauschka added an extra recognition sequence to the exogenous gene to give a different gene construction.

Hauschka has also been studying the FGF receptor and is presently trying to purify it. He has found that as FGF is removed from the culture medium, there is a loss of FGF receptors and apparently new receptors are not synthesized. The cells then go to terminal differentiation and become postmitotic. Hauschka has some preliminary evidence that the postmitotic state is regulated by a diffusible factor.

Transferrin as a Trophic Factor

T. Oh (University of Maryland, Baltimore) has been studying transferrin as a trophic factor and its receptor in developing chick skeletal muscle. He used dissociated embryonic chick skeletal muscle plated on collagen for his culture assay. After 7 to 12 days the embryonic cells develop into skeletal striated muscle followed by degeneration. Oh had found that the addition of an extract of CNS tissue to myoblasts in culture decreased the time for maturation to about 5 days. An extract of chicken sciatic nerve also increased the time for terminal differentiation of the cultures and prevented the degeneration of skeletal muscle. Oh isolated a factor of 84,000 molecular weight from chick sciatic nerve. It was an acidic protein with an IP of 5.75 which he termed sciatin. However, comparison of the amino acid sequence plus other physical-chemical characteristics with transferrin in addition to immunoreactivity with transferrin antibody proved conclusively that sciatin and transferrin (isolated from serum) were the same protein. Transferrin had been found to be a mediator of iron transport into the cell. However, Oh's studies using immunohistochemical procedures have shown transferrin is present in axons, ventral horn motor neurons, and spinal cord neurons. Transferrin has also been shown to have growth-promoting effects on cells in culture. Oh has found the presence of the 56,000 molecular weight transferrin receptor on the surface of rapidly dividing sciatic nerve. It was purified from embryonic CNS tissue, and antibodies to the receptor were produced. If the transferrin receptors are blocked by specific antibody, differentiation is prevented. Transferrin immunoreactivity is present at 6 days of development in chick neurons with a maximum at 10 days and absence at the adult stage. Spinal cord neurons also show maximum transferrin reactivity at 10 days. Oh has not found evidence for synthesis of transferrin by neurons but mentioned that it had been reported that transferrin mRNA was present in choroid plexus and oligodendrocytes using a cDNA probe. Oh is carrying out experiments to find out if, indeed, transferrin mRNA is present in neurons because the presence of mRNA, if true, shows that synthesis of transferrin does take place in neurons. Further studies are also being carried out to find out the role of transferrin in differentiation of neurons and to determine whether it is also involved in muscle cell differentiation.

6 CONCLUSION

The workshop-conference on "Growth Factors in the Nervous System" brought together researchers from the UK, US, Italy, Switzerland, France, and West Germany for a focused and productive meeting to exchange the most recent research in this important area of neurobiology. Much of the research consisted of unpublished work as well as preliminary data, which contributed greatly to the information exchange. It was evident that several new growth factors have been found and that some supposedly new factors might actually be factors already known. This aspect was also important, as the investigators were able to make arrangements with each other to test whether or not a "new factor" was indeed a previously unknown one. There was also evidence that glial factors play a role in neuronal differentiation and growth as well as nerve regeneration in addition to factors produced by the neurons. The effect of gangliosides and other cell-surface markers such as N-CAM in neurite extension and neuronal recognition indicates that multiple factors are involved in nerve growth. Undoubtedly, additional growth factors will be found, but the important question as to how they act is still not clearly defined. The use of molecular probes obtained by recombinant DNA methods has already provided some evidence, as shown in these presentations.

7 APPENDIX 1: LIST OF PARTICIPANTS

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8 APPENDIX 2: SCIENTIFIC PROGRAM

"The Role of Astrocyte-derived Growth Factors in Controlling the Time of Oligodendrocyte Differentiation," M. Raff.

"The Role of the Glial Environment in Promoting and in Inhibiting Axonal Growth in the Vertebrate CNS," J. Silver.

"Intercellular Communication Between Astrocytes, T cells, and Macrophages within the Brain," A. Fontana.

"Division and Differentiation in Normal and Neoplastic CNS," M. Noble.

"Characterization and Properties of Brain-derived Neurotrophic Factor (BDNF)," Y. Barde.

"Purification and Characterization of a Neurotrophic Factor from Bovine Striatum," A. Leon.

"Developmental and Regional Differences in the Growth Factor Requirement of Sensory Neurons," R. Lindsay.

"Nerve Growth Factor (NGF) Activated Gene Expression in PC12 Cells," G. Dickson.

"Factors Affecting Nerve Cell Survival and Neurite Outgrowth," L. Reichart (presented by D. Shelton).

"Proteins Influencing the Survival and Growth of Ciliary Ganglion Neurons," M. Manthorpe.

"Neurite-Promoting Factors for Spinal Neurons," C. Henderson.

"A Role for Cell-Surface Carbohydrates in Neuronal Recognition," R. Schnaar.

"Specificity of Ganglioside Effects on Neuritogenesis," P. Doherty.

"Directing Regeneration of Peripheral Motor Axons in the Frog," D. Kuffler.

"The Target Dependence of Normal Nissle Body Ultrastructure as Studied in Cat Intercostal Motor Neurons," T. Sears.

"Multiple Factors Controlling Nerve Growth in Muscle," M. Brown.

"Regulation of N-CAM (Neural Adhesion Molecule) Expression in Skeletal Muscle," F. Walsh.

"The Regulation of Skeletal Muscle Growth and Differentiation by Fibroblast Growth Factor," S. Hauschka.

"Transferrin and its Receptor in Developing Chick Skeletal Muscle and Neurons," T. Oh.

"Purification and Characterization of a Neurite Extension Factor from Bovine Brain Relationship to S-100 Protein," D. Kligman.

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